Oxidative stress and XIAP signalling in neuronal cells

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CONTENTS

ABBREVIATIONS

AD	Alzheimer's disease
ADP	adenosine diphosphate
AIF	apoptosis-inducing factor
AMPK	adenosine mono phosphate-activated kinase
ATP	adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl	B-cell lymphoma
BDNF	brain-derived neurotrophic factor
BH	Bcl-2 homology
BIR	baculovirus IAP repeat
CDNF	conserved dopamine neurotrophic factor
cIAP	cellular Inhibitor of Apoptosis Protein
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREB	cyclic adenosine monophosphate response element-binding
DHE	dihydroethidium
EGFP	Enhanced Green Fluorescent Protein
FasR	Fas Receptor
GDNF	glial cell line-derived neurotophic factor
GLF	glial cell line-derived neurotophic factor family ligand
GPx	glutathione peroxidase
GSR	glutathione reductase
ER	endoplasmic reticulum
HD	Huntington's disease
hILP	human IAP-like Protein
HTT	*huntingtin* (gene)
htt	huntingtin (protein)
IAP	Inhibitor of Apoptosis Protein
ICE	interleukin-1-beta converting enzyme
IκB	inhibitor of kappa B
IKK	inhibitor of kappa B kinase
IL	interleukin
IMS	intermembrane space
IT15	interesting transcript 15
JNK	c-Jun N-terminal Kinase
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MKK3	mitogen-activated protein kinase kinase kinase
MOMP	mitochondrial outer membrane permeabilisation
NAIP	Neuronal Apoptosis Inhibitor Protein
NFκB	Nuclear Factor kappa B
NGF	nerve growth factor
NT	neurotrophin
Omi/HtrA2	Omi / High-temperature requirement serine protease A2

PARP	poly-ADP-ribose polymerase
PD	Parkinson's disease
PGC1α	Peroxisome proliferator-activated receptor Gamma Co-activator 1 alpha
PNS	peripheral nervous system
$p75^{NTR}$	p75 neurotrophin receptor
RING	Really Interesting New Gene
RIP-1	receptor-interacting protein 1
ROS	reactive oxygen species
RSV	resveratrol
SIRT-1	silent information regulator 1
Smac/DIABLO	Second mitochondria-derived activator of caspase / Direct Inhibitor of Apoptosis-Binding protein with LOw pI
SOD	superoxide dismutase
TAB1	TGF-beta activated kinase 1 binding protein 1
TAK1	TGF-beta activated kinase 1
TG	transgenic
TNFα	tumour necrosis factor alpha
TNFR	tumour necrosis factor receptor
Trk	tropomyosin-related kinase
TRX	thioredoxin
TRXR	thioredoxin reductase
UBC	ubiquitin-conjugating
UPS	ubiquitin proteasome system
X	xanthine
XO	xanthine oxidase
XIAP	X-linked Inhibitor of Apoptosis Protein

ABSTRACT

Oxidative stress is the result of an imbalance in the cellular pro-/antioxidant homeostasis which leads to the overproduction of reactive oxygen species (ROS). Usually, oxidative stress leads to structural and functional disruption within the cells, which in turn may cause cell death through different mechanisms. The brain is very sensitive to oxidative damage because of its high oxygen consumption, and oxidative stress is known to be involved e.g. in neurodegenerative diseases such as Huntington's disease (HD).

X-linked Inhibitor of Apoptosis Protein (XIAP), one of the best-characterized apoptosis inhibitor proteins, is expressed both during development, and in the adult brain. Overexpression of XIAP is known to protect cells against various injuries and cell degeneration. XIAP can prevent cell death through various signalling cascades, e.g. by preventing caspases which are essential for apoptotic cell death. The function of XIAP in oxidative stress-related reactions is not that well-studied, which led us to examine it more closely. We found out that XIAP is able to reduce oxidative stress and cell death caused by ROS *in vitro* in neuron-like PC6.3-cells. We also noticed that XIAP increases the activation of transcription factor NFκB in these cells. Via NFκB, XIAP also increased two mitochondrial antioxidants: superoxide dismutase 2 (SOD2) and thioredoxin 2 (TRX2), and the significance of NFκB in this effect was also shown by using fibroblast cell lines from NFκB p65-/- mice. Furthermore, we observed that XIAP enhances BDNF signalling through NFκB in E17 HC neurons. In the PC6.3-cell model of HD there was a correlation between the increased amount of ROS and the enhanced number of glutamine repeats. The protein levels of intracellular antioxidants and NFκB were decreased in cells expressing mutant huntingtin (htt) proteins compared to control cells. Last but not least, we observed that resveratrol (RSV) pre-treatment is able to reduce oxidative stress and cell death caused by ROS in PC6.3-cells. RSV also increased the amount of SOD2, TRX2 and XIAP, and the NFκB system may be involved in the increase of antioxidant proteins mediated by RSV.

Altogether, these results bring us closer to understanding the role of XIAP in the control of oxidative stress and the function of XIAP in NFκB and BDNF signalling. In view of this, modulation of XIAP is an interesting possibility to consider in various therapies to reduce cell injuries caused by enhanced oxidative stress. RSV would also be a really interesting compound to examine as a drug candidate in disorders where oxidative stress is involved. However, studies to analyse its kinetics, actions and pharmacological and toxicological profiles are still lacking.

ORIGINAL PUBLICATIONS

This thesis is based on four original articles, which are referred to in the text by their Roman numerals (I-IV).

I **Kairisalo M**, Korhonen L, Blomgren K, Lindholm D. (2007) X-linked inhibitor of apoptosis protein increases mitochondrial antioxidants through NF-kappaB activation. *Biochem Biophys Res Commun*. 364(1):138-144.

II **Kairisalo M**, Korhonen L, Sepp M, Pruunsild P, Kukkonen JP, Kivinen J, Timmusk T, Blomgren K, Lindholm D. (2009) NF-kappaB-dependent regulation of brain-derived neurotrophic factor in hippocampal neurons by X-linked inhibitor of apoptosis protein. *Eur J Neurosci*. 30(6):958-966.

III Reijonen S, Kukkonen JP, Hyrskyluoto A, Kivinen J, **Kairisalo M**, Takei N, Lindholm D, Korhonen L. (2010) Downregulation of NF-kappaB signalling by mutant huntingtin proteins induces oxidative stress and cell death. *Cell Mol Life Sci*. 67(11):1929-1941.

IV **Kairisalo M**, Bonomo A, Mudò G, Belluardo N, Korhonen L, Lindholm D. Resveratrol reduces oxidative stress and cell death and increases mitochondrial antioxidants and XIAP in PC6.3-cells. (submitted)

In addition, some supplementary, unpublished data are presented.

1. REVIEW OF THE LITERATURE

1.1 General features of cell death

Kerr and coworkers (Kerr et al., 1972) divided cell death types into two categories according to their morphological structures: apoptosis and necrosis. Studies within past few years have also represented a third mechanism for cell death called necroptosis (Degterev et al., 2005, Teng et al., 2005b).

Apoptosis is an active, regulated cell death process and it is required during development and for homeostasis of an organ system. Activation of caspases (cysteine-aspartic acid proteases) has a leading role in apoptotic cell death, and some caspases are also required in the immune system. One of the primary contributors in tumour development and autoimmune diseases is deficiency of apoptosis, whereas unwanted apoptosis is occuring in ischemia and neurodegenerative diseases. Necrosis is a passive, pathological process and it is caused by extrinsic factors like infection or toxins. Necrosis is typically characterized by disruption of cell membranes and activation of the immune system.

Recent reports have described a caspase-independent but programmed and active cell death process called necroptosis (Degterev et al., 2005, Teng et al., 2005b). Necroptosis is somewhat in between apoptosis and necrosis. In some studies, necroptosis has also been connected to oxidative stress and production of reactive oxygen species (ROS) (Tait and Green, 2008, Cauwels et al., 2003). I have summarized the comparison of apoptosis, necrosis and necroptosis in figure 1. and table 1.

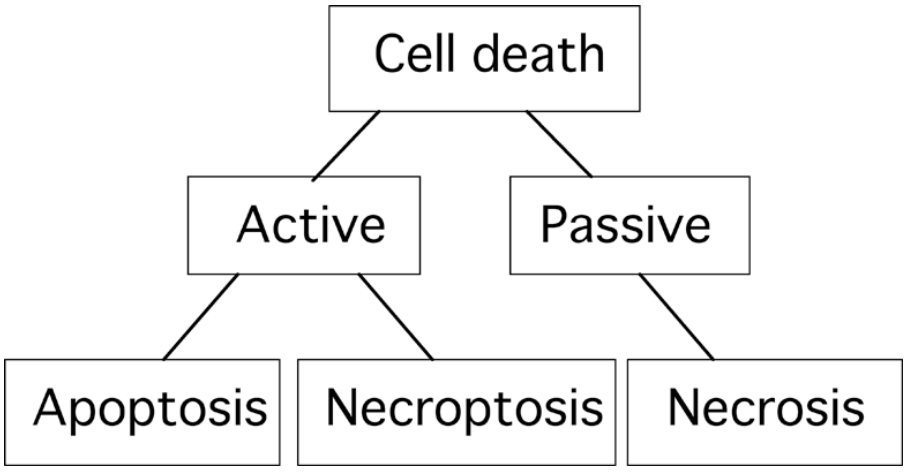

Figure 1. Division of cell death types.

Table 1. Main similarities/differences between apoptosis, necroptosis and necrosis.

Apoptosis	**Necroptosis**	**Necrosis**
Caspase-dependent	Caspase-independent	Caspase-independent
Active process	Active process	Passive process
Programmed	Programmed	Pathological, activates the immune system
Nuclear chromatin condensation and fragmentation	Partial nuclear chromatin condensation, no fragmentation	Chromatin clumping
Cleavage of caspase targets in mitochondria	Swelling of cristae	Mitochondrial disruption & dysfunction
Rapid loss of mito-chondrial memrane potential	Gradual loss of mito-chondrial memrane potential	
Blebbing of plasma membrane	Lacerated plasma membrane	Lacerated plasma membrane

1.1.1 Molecular mechanisms of different types of cell death

1.1.1.1 Death receptor pathways

Death receptors are cell surface receptors, which can mediate cell death through binding with the specific ligands. This pathway is also called the "extrinsic cell death pathway" (Loh et al., 2006). Death receptors mainly belong to the tumour necrosis factor receptor (TNFR) family, and e.g. Fas and p75 neurotrophin receptors ($p75^{NTR}$) are two well-known members in this group (Zampieri and Chao, 2006). Both of these receptors can activate the upstream caspase, caspase-8 cascade, and may also mediate the activation of Nuclear Factor κB (NFκB) and c-Jun N-terminal Kinase (JNK) (Barnhart et al., 2004, Kreuz et al., 2004, Waterston and Bower, 2004, Sancho-Martinez and Martin-Villalba, 2009). Especially NFκB is a pathway which is going to reappear later in this thesis.

Death receptors triggered apoptosis is well-characterized. However, recently it has been reported that death receptors can initiate caspase-independent, necroptotic cell death as well. It has been shown that mice treated with TNF together with caspase inhibitor died very fast, causing a huge upregulation of reactive oxygen species (ROS) (Cauwels et al., 2003). Necroptosis can also be induced by Fas (Tait and Green, 2008). Because necroptosis is linked to cell death caused by oxidative stress, it is worth concentrating on here.

Receptor-interacting protein-1 (RIP-1) kinase has an important function in death receptor-induced necroptosis (Tait and Green, 2008, Kelliher et al., 1998, Holler et al., 2000). It interacts both with the Fas-receptor (FasR), and the TNF-receptor (TNFR) (Hsu

et al., 1996). Activation of pro-survival protein NFκB also requires the intermediate domain of RIP-1, and that is why it cannot inhibit necroptosis of cells stimulated by TNFα without RIP-1 (Ting et al., 1996). One possible mechanism behind RIP-1 mediated necroptosis is TNFα-induced, RIP-dependent inhibition of adenosine diphosphate (ADP) -transportation into mitochondria, which will result in reduced adenosine triphosphate (ATP) and cell death (Tait and Green, 2008, Temkin et al., 2006). This further leads to mitochondrial dysfunction, which is known to be the cause of ROS production (Galluzzi and Kroemer, 2008). Another mechanism by which RIP-1 can mediate necroptotic cell death is reduction of caspase-8. Cells normally require a small amount of caspase-8 to stay viable, and reduction of caspase-8 expression induces necroptotic cell death (Yu et al., 2004). This is RIP-1- and JNK -dependent and comes up through (macro)autophagy. Autophagy (or autophagocytosis) is a cellular process which regulates degradation of elements of cells through lysosomal machinery. It has a role in protection of organisms in versatile pathological conditions like infections, cancer and neurodegeneration (Levine and Kroemer, 2008). Recently, it has been expressed that TNFα can induce cell death through two separate caspase-8 activation pathways which are regulated by cellular Inhibitor of Apoptosis Protein 1/2 (cIAP1/2) and cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP) (Wang et al., 2008). The cIAP regulated pathway is RIP-1 kinase independent while c-FLIP mediated pathway requires RIP-1 kinase.

1.1.1.2 Mitochondrial pathway

Mitochondria are membrane-enclosed cell organelles which are required to produce energy in the cell by generating ATP, and are involved in processes like cell death and growth, cell signalling, differentiation and control of the cell cycle (McBride et al., 2006). Various damages can occur if there is some failure in these processes, i.e. mitochondrial outer membrane permeabilisation (MOMP) leads to releasing of some mitochondrial intermembrane space (IMS) proteins, and following this, disruption of mitochondrial function, and/or induction of cell death (Tait and Green, 2008). These IMS-proteins include cytochrome-c, apoptosis-inducing factor (AIF), Second mitochondria-derived activator of caspase / Direct Inhibitor of Apoptosis-Binding protein with LOw pI (Smac/DIABLO), and Omi / High-temperature requirement serine protease A2 (Omi/HtrA2) (Tait and Green, 2008, Du et al., 2000, Susin et al., 1999, Verhagen et al., 2000, Suzuki et al., 2001c). Mitochondria mediated cell death can also be called the “intrinsic cell death pathway”.

Both cytochrome-c and AIF have important roles in mitochondrial respiration. One of the key events in the mitochondrial pathway is the release of cytochrome-c into the cytosol. Lack of cytochrome-c can lead to a burst of ROS generation because of respiratory dysfunction (Mootha et al., 2001, Zhao et al., 2003, Ow et al., 2008). Lack of AIF on the other hand leads to inhibition of mitochondrial respiratory chain complex I activity and compensation of oxidative phosphorylation (Vahsen et al., 2004). IAP-antagonists

Smac/DIABLO and Omi/HtrA2 are normally localised in the mitochondria, but in the presence of apoptotic stimuli they are released into the cytosol (Vaux and Silke, 2003, Wilkinson et al., 2004a, Wilkinson et al., 2004b, Nakka et al., 2008). Smac/DIABLO and Omi/HtrA2 both have pro-apoptotic effects because they can bind to IAPs and promote cell death (Du et al., 2000, Vaux and Silke, 2003, Nakka et al., 2008).

1.1.2 Caspases and other pro-apoptotic proteins involved in cell death

Caspases (or cysteine aspartases) are intracellular proteases which are essential for the apoptotic cell death process in the most cells. The importance of caspases in apoptosis was established when it was found that the cell death abnormal-3 (ced-3) gene was required for the cell death during the development of *Caenorhabditis elegans (C. Elegans)*. *C. Elegans* is a powerful model to study cell death pathways because there is a remarkable degree of conservation in apoptotic pathways of nematodes and mammals (Ellis and Horvitz, 1986, Peden et al., 2008, Metzstein et al., 1998). In this nematode, 131 of it's 1091 cells die via apoptosis (Ellis and Horvitz, 1991). *C. Elegans* has at least 15 genes which are involved in programmed cell death, and they can be divided into four groups according to their functions: 1) Decision-making (ces (cell death specification) -1 and -2)); 2) Execution (ced-3, -4, -9, and egl (egg laying defective) -1); 3) Engulfment of dying cells (ced-1, -2, -5, -6, -7, -10 and -12); and 4) Degradation of cell corpses (nuc (nuclease) -1).

It was found in 1993 that ced-3 encodes a member of caspase-family with similar properties to the mammalian interleukin-1-beta converting enzyme (ICE) (now known as caspase-1), which was the first known caspase (Yuan et al., 1993). Caspases can be divided into groups in different ways. One common way is to divide them into initiator (apical) caspases, and effector (executioner) caspases (Salvesen and Riedl, 2008). Initiator caspases (-2, -8, -9, and -10) are activated in response to apoptotic stimuli, and they cleave inactive pro-forms of effector caspases. This leads to activation of effector caspases (i.e. -3, -6, and -7), and they in turn cleave other protein substrates within the cell, triggering the apoptotic process. Another way is to divide caspases according to their substrate specificity (Thornberry et al., 1997). Group I caspases (-1, -4, -5, and -13) have hydrophobic amino acids in the position P4 of the cleavage site. In group II caspases (-2, -3, and -7) the aspartic acid (Asp; amino acid) is essential in the same position, and in group III caspases (-6, -8, and -9) there has to be an aliphatic amino acid in position P4. In addition to apoptosis during adult life and development, some caspases are also involved in the immune system. For example, caspase-8 is required for appropriate function of cell death cascades where cytokines like TNF and Fas/Apo1 are involved (Varfolomeev et al., 1998). Failure to undergo apoptosis is one of the main contributions to tumour development and autoimmune diseases, while the unwanted apoptosis occurs in ischemia and neurodegenerative diseases.

1.2 Anti- and pro-apoptotic proteins

Defects in regulation of cell death can occur in many diseases. Excessive cell loss is common to diseases like neurodegeneration, stroke, and heart failure; and cell accumulation occurs in diseases like cancer, autoimmunity, and inflammation (Jana and Paliwal, 2007).

1.2.1 B-cell lymphoma 2 (Bcl-2) family proteins

The *Bcl-2* proto-oncogene was first discovered in 1985 from human follicular B cell lymphoma (Bakhshi et al., 1985, Cleary and Sklar, 1985, Tsujimoto et al., 1985). The family of Bcl-2 proteins forms a critical intracellular checkpoint in the intrinsic pathway of apoptosis (Danial and Korsmeyer, 2004). They control MOMP, and they can be either pro- or anti-apoptotic. The Bcl-2 family proteins can be structurally divided into three subfamilies: the Bcl-2, Bcl-2-associated X protein (Bax) and Bcl-2 homology (BH) 3-only. I will not go into Bcl-2 in greater detail, because I haven't concentrated on it in my studies.

1.2.2 Inhibitor of Apoptosis Protein (IAP) family

The Inhibitor of Apoptosis Protein (IAP) gene was identified in baculovirus infected *Spodoptera frugiperda* SF-21 insect cells in 1993 (Crook et al., 1993). IAP family members can inhibit apoptosis through different mechanisms of which inhibition of caspases is the most well-known. IAP family members can be divided into several groups based on the presence of baculovirus IAP repeats (BIR) domains at the amino terminus of the protein (Wei Y et al., 2008) (figure 2). There are three subtypes of BIR domains: BIR1, -2 and -3. At the carboxyl terminus some of the IAPs have Really Interesting New Gene (RING) zink-finger domain. Ubiquitin-conjugating (UBC) domain can also be found in proteins belonging to the IAP Family (Lewis and Holcik, 2005, Eckelman et al., 2006).

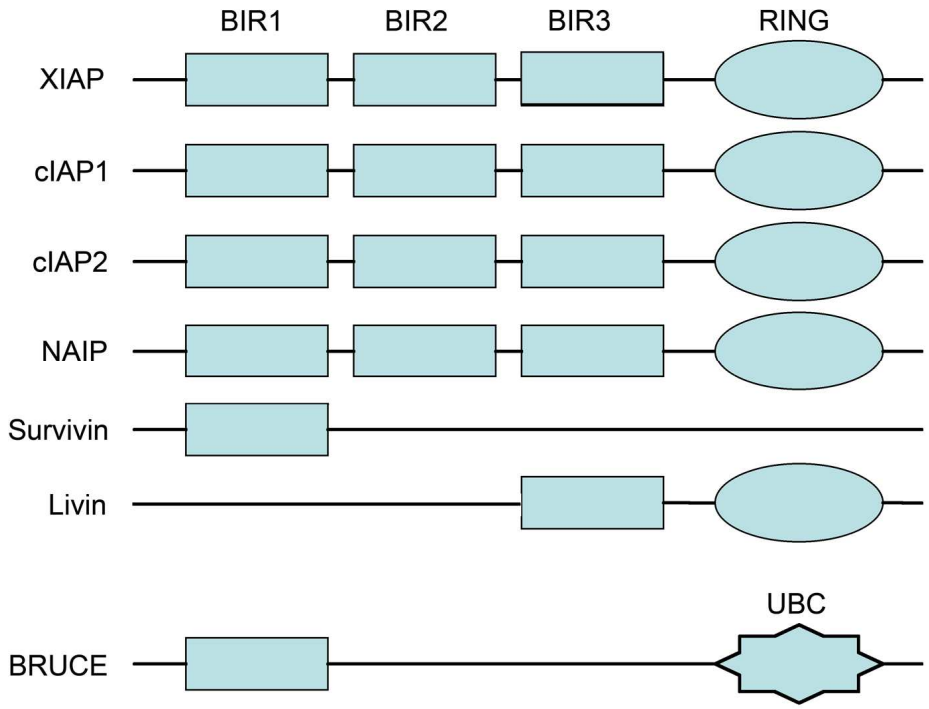

Figure 2. IAP family members can be divided according to their structures. XIAP, and Neuronal Apoptosis Inhibitor Protein (NAIP) all have three BIR domains and a RING domain. Survivin has only one BIR domain, and Livin one BIR domain and a RING domain. BRUCE, a giant protein member of this family, has one BIR domain and an UBC domain.

1.2.2.1 X-linked Inhibitor of Apoptosis Protein (XIAP)

XIAP (also called as baculoviral IAP repeat-containing 4 (BIRC4), or human IAP-like Protein (hILP); 57 kDa) is the most-well characterized protein among IAPs. It is expressed both during development and in the adult brain (Korhonen et al., 2001). As the name suggests, *XIAP* gene is located in the X chromosome. It has been shown in different cell types that overexpression of XIAP protects against various injuries and cell degeneration (Holcik et al., 2001, Perrelet et al., 2002, Trapp et al., 2003). XIAP prevents cell death through various signalling cascades (figure 3), and it has the ability to effectively prevent cell death caused by TNF-α, Fas, UV light, and genotoxic agents (Duckett et al., 1998).

XIAP contains three N-terminal BIR domains and a C-terminal RING domain (figure 4). BIR1 can interact with TGF-beta (β) activated kinase 1 (TAK1) binding protein 1 (TAB1) which is crucial for XIAP-induced activation of NFκB and TAK1 (Lu et al., 2007). TAK1 is a mitogen-activated protein (MAP) kinase kinase kinase (MKK3), and it activates NFκB and JNK-p38 kinase pathways by directly phosphorylating the inhibitor

of kappa B kinase (IKK) and MKK6 (Wang et al., 1998). NFκB is a transcription factor which has several functions in the body, e.g. it regulates genes which control cell survival and proliferation, and it plays a key role in regulating the immune response to infection. Both NFκB and JNK-p38 mitogen-activated kinases are responding to stress stimuli such as cytokines or ultraviolet irradiation. The JNK-p38 system is also involved in T cell differentiation and apoptosis.

BIR2 and BIR3 domains of XIAP are essential for the prevention of cell death caused by particular caspases. BIR2 domain of XIAP, together with the linker region between BIR1 and BIR2, are responsible for inhibition of caspase-3 and -7 (Chai et al., 2001, Huang et al., 2001, Riedl et al., 2001, Suzuki et al., 2001b). Via BIR3 XIAP prevents activation of caspase-9 and a mitochondrial protein called Smac/DIABLO (Chai et al., 2000, Liu et al., 2000, Srinivasula et al., 2001, Shiozaki et al., 2003). Binding of Smac/DIABLO to IAPs promotes e.g. activation of pro- and mature caspase-3 (Chai et al., 2000).

The main function of RING finger domain is to mediate autoubiquitinylation and ubiquitinylation of other proteins (Freemont, 2000, Richter and Duckett, 2000, Yang et al., 2000, Vaux and Silke, 2005). Ubiquitinylated proteins are labelled for proteasomal degradation. Ubiquitinylation controls the stability, function and intracellular localisation of several proteins. Ubiquitin ligase activity of XIAP enhances its cell death preventing effects in Fas-induced cell death, and deletion in the RING domain sensitizes MEFs to TNFα (Suzuki et al., 2001a, Schile et al., 2008). This indicates that ability of XIAP to inhibit the activation of death receptor signalling depends at least partly on the presence of the RING domain.

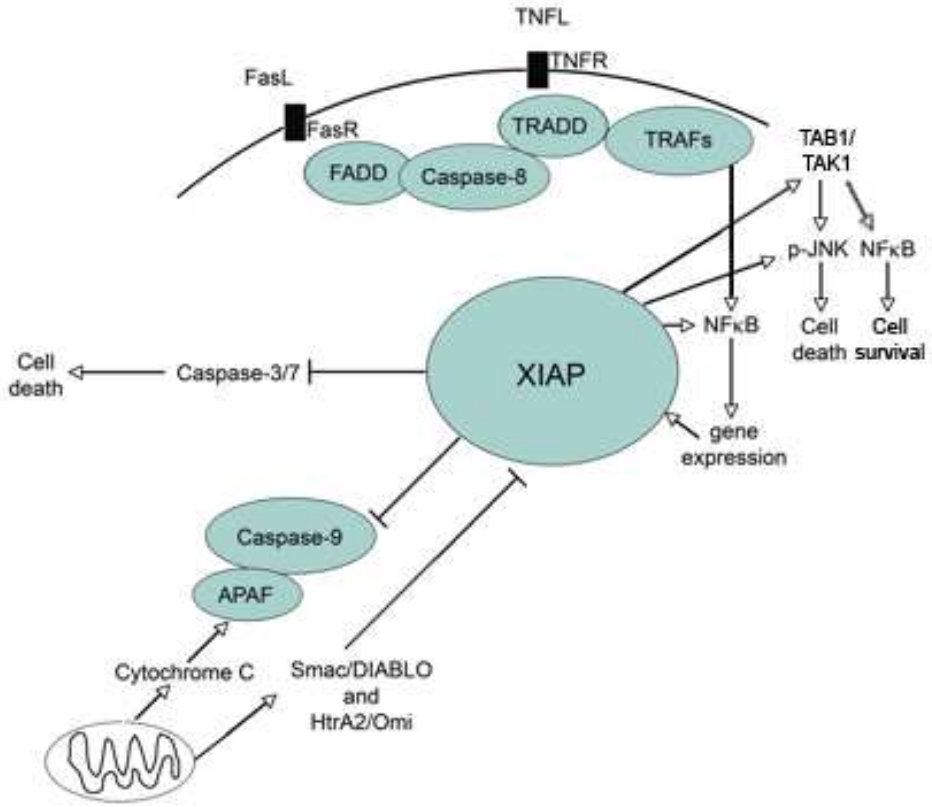

Figure 3. XIAP prevents cell death through various signalling cascades. The most well known mechanism is the ability of XIAP to inhibit caspases. Through caspase-9 and Apoptotic Protease Activating Factor (APAF), XIAP can modulate the mitochondrial cell death pathway, while Smac/DIABLO and HtrA2/Omi have opposite effects on cell death compared with XIAP. Via JNK and NFκB XIAP is able to modulate cell death and the transcription of other genes involved in cell death. Through TAK1 XIAP is also connected to death receptor signalling.

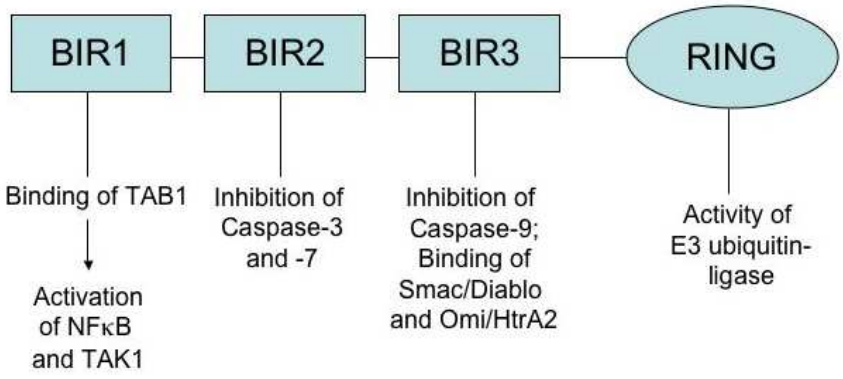

Figure 4. The structure of XIAP has three BIR domains and a RING domain. XIAP is able to prevent various types of cell death and each domain of XIAP can affect cell death processes in different ways. Some of the linker regions between domains are required for the actions of XIAP as well.

1.3 Oxidative stress and intracellular antioxidant enzymes

Molecular oxygen is used for many cellular reactions such as catalysis and energy production. These reactions in turn produce ROS, which are normal by-products of cellular metabolism and normally eliminated by intracellular antioxidant enzymes. Oxidative stress is the result of an imbalance in the cellular pro-oxidant/antioxidant homeostasis which leads to the overproduction of ROS, and it can follow e.g. from environmental factors, neurodegeneration or inflammation (Barnham et al., 2004). Oxidative stress can cause mitochondrial dysfunction such as reduced ATP production, decreased ubiquitin proteasome system (UPS) function, and impaired calcium homeostasis. Structural and functional disintegration of cells, induced by overproduction of ROS, can be mediated through various mechanisms like lipid peroxidation, sulfhydroxyl oxidation, proteolysis, and shearing of the nuclear material (Loh et al., 2006). These in turn cause cell death by apoptosis, necroptosis or necrosis. In the past, direct treatment with oxidants was thought to induce solely necrosis. However, later studies showed that lower doses of free radicals can trigger apoptosis as well, and more recent studies have considered the purpose of necroptosis in cell death caused by oxidative stress (Tait and Green, 2008, Cauwels et al., 2003, Loh et al., 2006). The contribution of ROS in different cell death types is highly cell-type specific, and also depends on the amount of endo- or exogenously generated ROS present (Nakano et al., 2006). ROS can induce apoptosis through a mitochondria-dependent pathway mediated by activation of JNK, or it can act directly on the mitochondria resulting in the release of apoptotic factors such as cytochrome-c, AIF, or Smac/DIABLO. Necrosis (and necroptosis) caused by ROS can be mediated through death receptor induced pathways, and it is typical during overproduction of ROS that JNK is chronically activated (Nakano et al., 2006, Sakon et al., 2003, Nakano, 2004, Nakano, 2005).

The brain is very sensitive to oxidative damage because of its high oxygen consumption. It is known that oxidative stress is involved e.g. in neurodegenerative diseases such as Huntington's, Parkinson's, and Alzheimer's diseases, and in inflammatory diseases like inflammatory joint disease (Barnham et al., 2004, Henrotin et al., 2003, Rego and Oliveira, 2003, Afonso et al., 2007). It is not fully clear, whether the overproduction of ROS is a cause or a consequence in these diseases. Anyhow, ROS can damage the respiratory components which further increase the production of free radicals, and that way potentiate the whole damaging effect.

1.3.1 Elimination of ROS with intracellular antioxidant scavenger enzymes

ROS are small molecules or atoms which have unpaired valence shell electrons, and they rapidly accept another electron or transfer the unpaired electron to another molecule. ROS are mainly developed in mitochondria (Reddy, 2006). Under normal conditions, there is a balance between pro- and antioxidant proteins within the cells, where increased

levels of ROS are counteracted by antioxidant systems including superoxide dismutases (SODs), and thioredoxins (TRXs).

SODs are intracellular antioxidants which catalyse the reaction of a highly reactive oxygen radical called the superoxide anion (O_2^-) to alter dioxygen (O_2) and hydrogen peroxide (H_2O_2):

$$\mathbf{2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2}$$

SODs are named or numbered according to their location within cell organelles:

1. SOD1 can be found in the cytoplasm, nucleus and intermembrane space of mitochondria. It contains copper (Cu) and zinc (Zn), which is why it is also called Cu/Zn-SOD. Expression of SOD1 can be inhibited e.g. by tumour necrosis factor –α (TNFα) and this explains why there is O_2^- and derivatives in inflammatory conditions (Afonso et al., 2006). TNFα inhibits SOD1 through the JNK/AP-1 pro-apoptotic pathway.
2. SOD2 is located in mitochondria. It uses manganase (Mn) as a cofactor, and it can be also called Mn-SOD. SOD2 can be activated e.g. by pro-inflammatory cytokines such as interleukin-1, -4, and -6 (IL-1, IL-4 and IL-6), and TNFα. The promoter region involved in activation of SOD2 contains sites that bind to transcription factors belonging to the families of nuclear factor κB (NFκB), CCAAT/enhancer-binding protein (C/EBP) and nuclear factor-1 (NF-1) (Kiningham et al., 2001).
3. SOD3 is an extracellular protein (EC-SOD), and it also uses Cu and Zn as cofactors - same way as SOD1. I will not go into this in more detail, because in our studies we have investigated mainly the intracellular SOD enzymes.

Hydrogen peroxide, which has been generated after reactions mentioned above, is also a pro-oxidant, although not as reactive as O_2^-. H_2O_2 can further be eliminated by an intracellular antioxidant enzyme catalase to water (H_2O) and molecular oxygen (O_2):

$$\mathbf{2H_2O_2 \rightarrow O_2 + 2H_2O\ (catalase)}$$

Catalase is a heme-containing enzyme localised mainly in the peroxisomes (Nordberg and Arner, 2001, Maulik and Das, 2008). H_2O_2 can also be eliminated by the intracellular antioxidant enzyme glutathione peroxidase (GPx). There are several isozymes of GPx, which have various cellular locations and substrate specificities. GPx1 is the most abundant version, and it is localised in the cytoplasm. The way it converts H_2O_2 to water differs from the way catalase catalyses that reaction:

$$\mathbf{2GSH + H_2O_2 \rightarrow GSSG + 2H_2O\ (GPx)}$$

In this reaction GSH represents a monomeric glutathione and GSSG glutathione disulfide. After this reaction an enzyme called glutathione reductase (GSR) completes the cycle by reducing the oxidized glutathione:

$$\mathbf{GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+\ (GSR)}$$

A less well studied enzymatic intracellular antioxidant system is the thioredoxin system, which consists of thioredoxin (TRX), thioredoxin reductase (TRXR), and NAPDH (Maulik and Das, 2008). These TRX family proteins take part in redox reactions and in cellular defense against ROS (Takagi et al., 1999, Patenaude et al., 2004). There are two main isoforms of TRX protein: TRX1 is a “classical”, cytosolic, and the most studied form of TRXs; and TRX2 is a less studied protein with mitochondrial translocation

(Nordberg and Arner, 2001, Maulik and Das, 2008, Spyrou et al., 1997, Taniguchi et al., 1996). TRX system is highly effective in reducing disulfides of other proteins and peptides:

$$\mathbf{TRX\text{-}(SH)_2 + Protein\text{-}S_2/Substrate_{oxized} \rightarrow TRX\text{-}S_2 + Protein\text{-}(SH)_2/Product_{reduced}}$$

The oxidised TRXs are reduced and kept in a reduced state by TRXR, which completes the cycle by reducing the active site disulfide of TRX:

$$\mathbf{TRX\text{-}S_2 + NADPH + H^+ \rightarrow TRX\text{-}(SH)_2 + NADP^+}$$

Altogether, by influencing intracellular antioxidant proteins, it could be possible to improve the state of oxidative stress as well.

1.4 Transcription factor Nuclear Factor kappa B (NFκB)

NFκB is a nuclear transcription factor that was first identified in 1986 (Sen and Baltimore, 1986). It was named according to its localisation when it was first found: in the nucleus, bound to an enhancer element of immunoglobulin κ light chain gene in B cells. NFκB is expressed almost in all animal cell types, and it is involved in several cellular responses to various stimuli, such as stress, cytokines and free radicals. Conversely, incorrect regulation of NFκB has been linked to various diseases, i.e. cancer, inflammatory diseases, and neurodegenerative diseases.

1.4.1 Structure of NFκB

All proteins of the NFκB/Rel family share a Rel homology domain (RHD) in their N-terminus. There are five proteins in NFκB/Rel family: NFκB1 (p50 and its precursor p105), NFκB2 (p50 and its precursor p100), RelA (p65), RelB, and c-rel (Siebenlist et al., 1994, Karin and Ben-Neriah, 2000). Those can be further divided into two protein subfamilies (table 2). NFκB1 and NFκB2 proteins are synthesized as large precursors (p105 and p100), and they undergo the ubiquitin/proteasome system mediated process to generate the mature NFκB subunits (p50 and p52). This process involves selective degradation of their C-terminal region containing ankyrin repeats, while RelA, RelB, and c-Rel have a transactivation domain in their C-terminus.

Table 2. Classification of NFκB family proteins

Subfamily I	NFκB1/p105 → p50 NFκB2/p100 → p52
Subfamily II	RelA/p65 RelB c-rel

1.4.2 Activation of NFκB

All of the NFκB/Rel family proteins have a 300-amino acid Rel homology region, which is responsible for their main actions. They can form both homo- and heterodimers, and the most commonly known NFκB complex consists of p50/p65 heterodimer, which is also the most often used term to describe NFκB. The inactive state of NFκB is bound by inhibitory complex IκB, which holds it as a cytoplasmic protein complex. Upon stimulation, the IκB subunit is phosphorylated by IκB kinases (IKKα and IKKβ), and in the classic NFκB model IκBα is transiently degraded by UPS, with consequent release of dimers of p65/p50 to translocate into the nucleus (Hacker and Karin, 2006, Sethi et al., 2008) (figure 5). In the nucleus they bind to DNA target sites (known as "κB sites"), and directly regulate gene expression of numerous genes. This whole reaction is usually rapid (within the first hour after stimulation).

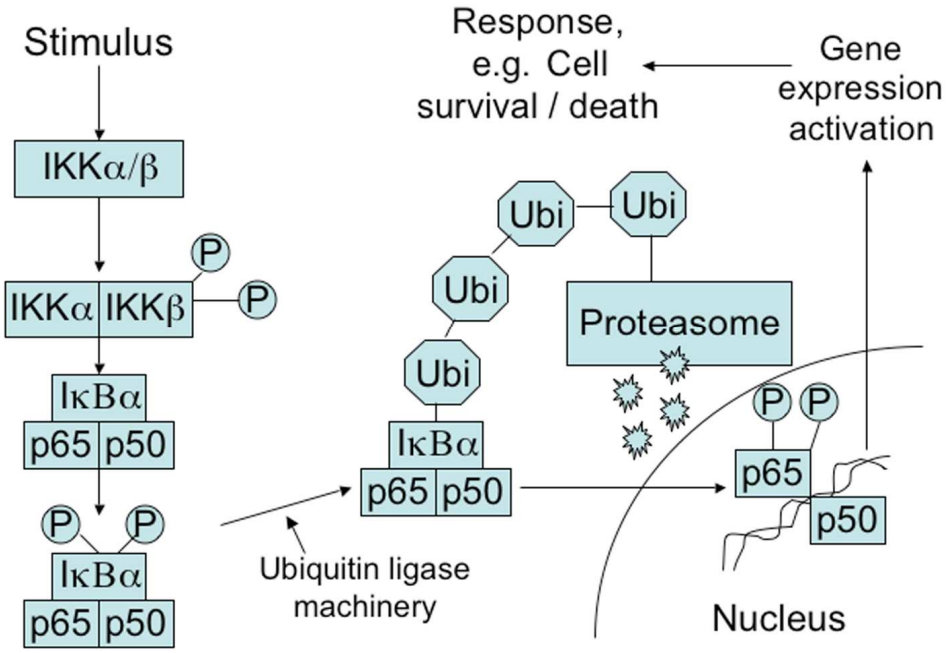

Figure 5. The most classic model of how the NFκB pathway can be activated.

Later it has been shown that NFκB activation is actually a biphasic reaction (Ladner et al., 2003, Caba and Bahr, 2004, Miskolci et al., 2007). The later phase can exist about ten hours after continuous stimulation, and activation can still exist even the following day. It is also possible that NFκB is persistently activated if stimulation is continuous (Miskolci et al., 2007). A common feature in to this "late-phase activation of NFκB" is that the activation can be IκBα-independent, or IκBα is only partly degraded (Ladner et al., 2003, Miskolci et al., 2007). Degradation of IκBβ can also be involved in the later phase (Ladner et al., 2003, Caba and Bahr, 2004). It is also probable that distinct phases of NFκB activation regulates different genes, of they even may lead to opposing cellular responses (Caba and Bahr, 2004).

1.5 Neurotrophic factors

Neurotrophic factors are growth factor proteins which have a numerous amount of functions in the central nervous system (CNS) and peripheral nervous system (PNS) in developing and adult mammals (Allen and Dawbarn, 2006). They are involved in different cell signalling processes like cell survival, differentiation and growth. The group of neurotophic factors includes: 1) neurotrophins (NTs) such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophins 3 and 4 (NT-3 and NT-4); 2) neurokines like ciliary neurotrophic factor (CNTF); and 3) glial cell line-derived neurotophic factor (GDNF) family ligands (GFLs) (Airaksinen and Saarma, 2002, Bespalov and Saarma, 2007). Recently a new neurotrophic factor called conserved dopamine neurotrophic factor (CDNF) was discovered in Helsinki (Lindholm et al., 2007). It differs from neurotrophins and GDNF structurally and functionally. CDNF has a neurotrophic activity specifically for brain neurons and particularly for dopaminergic neurons *in vivo*. Loss of neurotrophic factors and their activity have been connected to different neurodegenerative diseases. NGF and tropomyosin-related kinase (Trk) A receptor may play a role in Alzheimer's disease (AD), and BDNF in Huntington's Disease (HD). *In vivo* results suggest that both GDNF and CDNF could be powerful tools in the treatment of Parkinson's disease (PD) (Lindholm et al., 2007, Dawbarn and Allen, 2003).

1.5.1 Neurotrophins

Neurotrophins are all synthesized as precursors ("pre-pro-form"), and whereas pro-NGF, pro-NT-3, and pro-NT-4 are packaged into constitutive vesicles before secretion, pro-BDNF is mainly packaged into regulated secretory pathway vesicles, and secreted in an activity-dependent manner (Allen and Dawbarn, 2006, Mowla et al., 2001, Hibbert et al., 2003). The pre-region of the protein is cleaved during secretion in the endoplasmic reticulum (ER), and after that, the pro-from is cleaved in the Golgi or in the secretory

granules (Seidah et al., 1996, Thomas and Davies, 2005). At least pro-forms of BDNF and NGF are secreted without being cleaved and while mature forms of neurotrophins have usually neuroprotective properties, these pro-forms have mainly pro-apoptotic functions (Nykjaer et al., 2005).

Neurotrophins can act through two different receptor types: 1) the family of tyrosine kinase Trk receptors; and 2) the "pan" neurotrophin receptor called $p75^{NTR}$ (p75 neurotrophin receptor). Trk receptors are activated by binding of specific ligands to their extracellular domains. Activated receptors act like tyrosine kinases. This means that they are able to phosphorylate certain tyrosines, and so activate signalling processes within the cell. Trk receptors have various subtypes (A-C), and each of them binds to a spesific group of neurotrophins: TrkA binds NGF, TrkB binds both NT-4 and BDNF, and TrkC binds NT-3. $p75^{NTR}$ belongs to the TNFR-family, and all neurotrophins bind to it with similar affinity. Upon binding of pro-neurotrophins, $p75^{NTR}$ can activate apoptosis through its intracellular "death domain" (Nykjaer et al., 2004, Teng et al., 2005a).

Many investigations indicate that the survival-promoting signals of neurotrophins are generated by activation of Trk receptors, and that their death-promoting signals are generated by activation of $p75^{NTR}$ receptors (Kalb, 2005). There is also an assessment that mature forms of neurotrophins bind to Trk receptors promoting cell survival, and pro-forms to $p75^{NTR}$ receptors promoting cell death. Preferentially it goes this way, but there is evidence that pro- and mature forms of neurotrophins can also bind to both receptor types and activate survival-promoting and death-promoting pathways. Thus, NGF promotes the survival of sensory neurons by activation of NFκB, and this seems to be mediated by $p75^{NTR}$, and on the other hand, pro-neurotophins bind weakly to Trk receptors as well (Hamanoue et al., 1999, Lee et al., 2001). There are also indications for crosstalk between Trk and $p75^{NTR}$ receptor pathways: activation of $p75^{NTR}$ receptor may lead to enhance the affinity of neurotrophins to bind to the Trk receptors, and thereby promote positive signalling of neurotrophins (Zampieri and Chao, 2006, Kalb, 2005).

1.5.1.1 Brain-derived neurotrophic factor (BDNF)

BDNF is a well-known neurotrophic factor which promotes neuronal survival, growth and differentiation of new synapses and neurons, and induces neuronal plasticity (Acheson et al., 1995, Huang and Reichardt, 2001, Greer and Greenberg, 2008, Matsumoto et al., 2008). It is expressed in specific brain areas, such as hippocampus, cortex, and basal forebrain, which are essential to learning, memory, and cognition (Yamada and Nabeshima, 2003). BDNF also plays an important role in neural development and it is one of the most active neurotrophins in helping to control and stimulate neurogenesis (Ernfors et al., 1995, Zigova et al., 1998, Benraiss et al., 2001, Pencea et al., 2001).

The BDNF protein is coded by the *BDNF* gene which is located on chromosome 11 in humans (Jones and Reichardt, 1990, Maisonpierre et al., 1991). The BDNF protein is synthesized as a 36 kDa precursor protein which is post-translationally converted into 14 kDa, mature BDNF. Recent studies using mouse hippocampal neurons indicate that small amounts of pro-BDNF is also released from neurons (Matsumoto et al., 2008). Mature BDNF binds both to the TrkB and $p75^{NTR}$ receptors. and pro-BDNF can also bind to both receptors. It has been shown that pro-BDNF can induce apoptosis through the $p75^{NTR}$ and sortilin receptor complex (Teng et al., 2005a). In pathological conditions of the brain one possible reason for neuronal loss can be an overproduction of pro-neurotrophins (Volosin et al., 2006). Usually donwregulation of mature BDNF is also involved. BDNF has been linked to various diseaseses such as Huntington's Disease, Parkinson's Disease, Alzheimer's Disease and depression (Zuccato et al., 2003, Brunoni et al., 2008, Dwivedi, 2009, Zajac et al., 2009, Zuccato and Cattaneo, 2009).

1.6 Huntington's Disease (HD) and oxidative stress

1.6.1 Background of HD

HD has been named after George Huntington's description of this particular condition which is also known as *chorea* (the Greek word for dance) (Huntington G., 1872). Prevalence of HD in the whole world is about 5-10/100 000 (Landles and Bates, 2004). Patients are about 30-50 years old at the onset of disease and they usually die in 10-20 years after the first symptoms. Physical symptoms of HD are i.e. bradykinesia, clumsiness and defects in voluntary movements (Thompson et al., 1988, Gil and Rego, 2008). These are usually accompanied with cognitive and psychiatric symptoms like impaired executive functions or depression (Walker, 2007). Neuropathological changes of HD occur in various brain regions but the primary site of neurodegeneration is in the striatum (caudate and putamen) and specially the striatal medium spiny neurons are the most susceptible (Walker, 2007, Vonsattel and DiFiglia, 1998, Browne and Beal, 2006) The largest part of the cell death happens in striatum but it has been shown that cell death occurs also in cerebral cortex, hippocampus and thalamus (Vonsattel et al., 1985, Cudkowicz and Kowall, 1990, Hedreen et al., 1991, Spargo et al., 1993). Striatal atrophy begins a long time before HD is diagnosed.

HD is caused by the expansion of the repeat in a gene called *huntingtin* (HTT). HTT, also referred to as interesting transcript 15 (IT15), was discovered in 1993 and mutation in this gene is involved in pathogenesis of HD (A novel gene containing a trinucleotide repeat that is expanded and unstable on huntington's disease chromosomes. the huntington's disease collaborative research group. 1993). Exon 1 of the coding region of the HTT gene contains a DNA-segment known as a CAG trinucleotide (glutamine) repeat. Expansion of this repeat site in HTT gene and it is leading to the formation of intracellular and intranuclear aggregates of misfolded huntingtin protein (Zoghbi and Orr,

2000, Li and Li, 2004). Normal individuals have < 35 CAG repeats. However, alleles with 35-39 CAG repeats are connected with late onset HD, while 40-50 repeats are common in adult-onset and the longest (> 70) repeats are involved in the most severe, juvenile and infantile HD-cases (A novel gene containing a trinucleotide repeat that is expanded and unstable on huntington's disease chromosomes. the huntington's disease collaborative research group. 1993). In other words, the number of CAG repeats is associated with the age of onset of HD, which is also typical in other polyglutamine diseases. IT15 encodes a huge protein called huntingtin (htt), with molecular weight of 348 kDa. Wild-type huntingtin has an important role in normal embryonic development and neuronal survival (Reiner et al., 2003). It's localised in cytoplasm and organelles such as mitochondria, Golgi apparatus, endoplasmic reticulum, synaptic vesicles, and several components of the cytoskeleton but small amounts of it also occurs inside the nucleus (Gil and Rego, 2008, Hoogeveen et al., 1993, Kegel et al., 2002). Huntingtin has been implicated i.e. in transcriptional regulation, endocytosis and intracellular transportation.

1.6.2 The roles of oxidative stress and BDNF in HD

ROS, mitochondrial dysfunction and metabolic impairment are known to be involved in several neurodegenerative diseases (Trushina and McMurray, 2007, Imarisio et al., 2008). It still not clear, whether these are causes or consequences of the disease but results from both post-mortem brains of HD patients and transgenic mouse models of HD suggest that they could have an important role in HD pathogenesis (Imarisio et al., 2008, Mangiarini et al., 1996, Tabrizi et al., 1999). The length of CAG repeats of HTT is closely linked to mitochondrial function. Mitochondrial impairment and oxidative stress have even been discovered in asymptomatic carriers of HD (Saft et al., 2005). Studies in post-mortem tissue of HD patients have shown several other pathological changes in HD brain which can be connected to oxidative stress and metabolic and mitochondrial dysfunction (Gil and Rego, 2008). It has been shown that both mitochondrial ATP/ADP production and calcium handling decreases if the number of CAG repeats increases (Seong et al., 2005, Oliveira et al., 2006). This happens because impaired energy metabolism caused by enhanced CAG repeats leads to reduced mitochondrial membrane potential when mitochondria cannot handle calcium normally, and they will be more sensitive to calcium influx and excitotoxicity (Oliveira et al., 2006, Novelli et al., 1988, Fagni et al., 1994). In normal situations, oxidative stress should be in balance with antioxidant proteins of the body.

Huntigtin together with Huntingtin-Interacting Protein 1 (HIP1) and Huntintin-Associated Protein 1 (HAP1) have roles in endocytosis and vesicle transportation (Velier et al., 1998). Mutant huntingtin causes e.g. impaired transportation of vesicles, and one important change after this is attenuated BDNF transportation, which leads to deprivation of neurotrophic support (Gil and Rego, 2008, Gauthier et al., 2004). Loss of BDNF gene transcription mediated by mutant huntingtin has been demonstrated in an HD-model,

while wild-type huntingtin was shown to upregulate the transcription of BDNF (Zuccato et al., 2001, Cattaneo et al., 2005). Expression of BDNF is regulated by the protein repressor element-1 silencing transcription factor/neuron restrictive silencer factor (REST/NRSF) (Zuccato et al., 2003). In the normal situation, wild-type huntingtin binds to REST/NRSF, and the reduction of REST/NRSF activity leads to enhanced expression of BDNF but mutant huntingtin has a failure to bind to REST/NRSF, which is why transcription of BDNF is repressed. BDNF has a crucial role in cell survival of striatal neurons and in the activity of cortico-striatal synapses.

There is also a long list of other transcription factors which are known to interact with huntingtin. Cyclic AMP-response element binding (CREB) protein (CBP), nuclear factor –κB (NFκB) and p53 are few examples of transcription factors which are affected by CAG expansion (Steffan et al., 2000, Roze et al., 2008). Within past few years it has been noticed that mutant huntingtin can also prevent the expression of Peroxisome proliferator-activated receptor Gamma (γ) Co-activator 1 alpha (PGC1α) (Cui et al., 2006, Weydt et al., 2006). This leads to reduced expression of PGC1α mitochondriąl targeț genes such as antioxidant enzymes superoxidase dismutase 2 (SOD2) (St-Pierre et al., 2006). All the transcription factors or co-factors mentioned above are known to be somehow in connection with oxidative stress, cell survival and/or BDNF, which makes these pathways really interesting subjects to study.

1.7 Resveratrol is a potent candidate in the treatment of diseases where oxidative stress is involved

Resveratrol (3,4',5-trihydroxystilbene, RSV) is a polyphenol compound primarily found in grapes and red wine. RSV has a large range of biological actions including protection of cells against oxidative stress (Baur and Sinclair, 2006). The ability of polyphenolic compounds, including resveratrol, to act as antioxidants are related to redox properties of the phenylic hydroxyl groups and the potential for electron delocalisation across the chemical structure (de la Lastra and Villegas, 2007) (chemical structure of RSV in figure 6).

Already some ten years ago it was observed that RSV protected rat hearts from ischemic injury (Das et al., 1999, Ray et al., 1999). Later the capability of RSV to protect against ischemic brain damage was reported, and RSV could be useful in the treatment of chronic, neurodegenerative diseases like Huntington's, Parkinson's and Alzheimer's Disease as well (Parker et al., 2005, Anekonda, 2006, Wang et al., 2006, Okawara et al., 2007, Blanchet et al., 2008, Chao et al., 2008, Della-Morte et al., 2009).

The mechanisms behind the antioxidant properties of RSV are not clear. There are data showing that RSV increases the activities of adenosine mono phosphate (AMP)-activated kinase (AMPK), silent information regulator 1 (SIRT-1), and PGC-1α (Baur et al., 2006, Lagouge et al., 2006). These are all connected to energy metabolism of the cells. PGC1α has been shown to be induced together with several intracellular antioxidant enzymes (St-

Pierre et al., 2006). RSV is also able to affect survival pathways like NFκB and mitogen activated protein kinases (MAPKs) (Aggarwal et al., 2004, Kundu et al., 2004, Holme and Pervaiz, 2007). However, it is important to remember that the survival promoting effects of RSV depend on the concentration, as high concentrations of RSV can trigger apoptosis instead of survival (Jang et al., 1997, In et al., 2006). RSV could be a potentially interesting drug candidate to consider in various diseases, but the therapeutic window and mechanism of its action require to be determined.

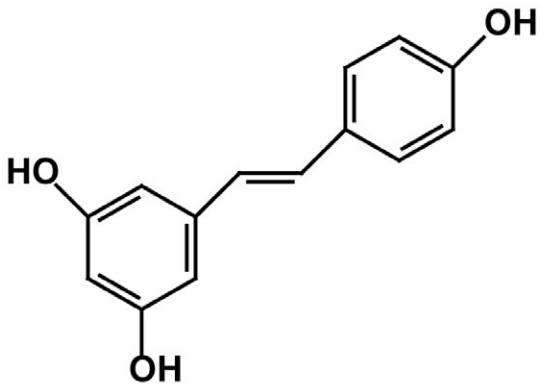

Figure 6. Chemical structure of RSV.

2. AIMS OF THE STUDY

The purpose of this thesis is to give novel insights into the ability of XIAP to reduce oxidative stress, and protect cells against cell death. We have also studied oxidative stress in the Huntington's disease model, and investigated the capability of resveratrol to reduce oxidative stress and cell death in neurons.

The specific aims of this thesis were:

- To investigate the possible role of XIAP in oxidative stress in neuronal cells (I, II)
- To study the effects of XIAP on NFκB and BDNF signalling (I, II)
- To identify the role of NFκB and oxidative stress in Huntington's Disease (III)
- To investigate if RSV is able to modulate oxidative stress in neurons, and clarify the mechanisms involved (IV)

3. MATERIALS AND METHODS

The experimental methods used in this thesis are listed in table 3, and short descriptions of them are given below the table. More detailed descriptions are found in the original publications.

Table 3. Methods used in this thesis.

Method	**Publication**
Cell culture, transfections and stimulations	I, II, III, IV
ROS measurement with FACSAria	I, III, IV
MTT assay	I, IV
Western Blotting	I, II, III, IV
Immunocytochemistry	I
Luciferase Assay	I, II, III. IV
PCR and light cycler	I, II, III, IV

3.1 Cell culture, transfections and stimulations

PC6.3-cells were cultured in RPMI 1640 medium supplemented with 10% Horse Serum (HS) and 5% Fetal Calf Serum (FCS). Swiss 3T3 and immortalized fibroblast cell lines from NFκB RelA/p65 knockout mice were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FCS. For overexpressing XIAP**,** both cell lines were transfected using expression plasmids encoding XIAP fused to Enhanced Green Fluorescent Protein (EGFP) (or in some experiments fused to myc) and TransFectin™ Lipid Reagent according to manufacturer's instructions (Bio-Rad). When XIAP-EGFP expression plasmid was used, control cells were transfected with EGFP expression vectors. To induce oxidative stress, PC6.3-cells were stimulated with xanthine sodium and xanthine oxidase (Valencia and Moran, 2004). Aspirin (acetylsalicylic acid (ASA)) was used in the first (I) publication because of it's ability to inhibit NFκB (D Acquisto F. et al., 2002). In some experiments of the supplementary data BAF (Boc-D-FMK) was used to inhibit caspases. In the third (III) article PC6.3-cells were transfected with expression plasmids encoding different polyQ repeats (18-120 Q) of huntingtin exon-1 fused to EGFP for modeling HD. In the fourth (IV) study we investigated the effect of resveratrol on oxidative stress in PC6.3-cells.

Hippocampal neurons were prepared from embryonic day 17 Wistar rats. E17 HC neurons were cultured in Neurobasal medium containing 2% B27, and transfected either with XIAP-myc expression plasmid or silencing RNA (siRNA) against XIAP using AMAXA Rat Neuron Nucleofector system. The efficiency of transfections was ensured by Western Blotting analysis by using specific antibody against XIAP. In certain experiments of the second (II) publication, neurons were stimulated with BDNF or IL-6. Embelin (2,5-dihydroxy-3-undecyl-1,4-benzoquinone) was used in some experiments to

antagonize XIAP (Nikolovska-Coleska Z et al., 2004), and recombinant TrkB/Fc chimeras to scavenge and inhibit BDNF action. Some of the cells also received an antibody against IL-6.

3.2 Measurement of ROS with FACSAria

Dihydroethidium (DHE) was added into the medium of PC6.3-cells for the last 15 minutes to indicate production. After this, cells were suspended into PBS and immediately examined by FACSAria (BD Biosciences). First EGFP-positive cells were found with excitation at 488 nm and emission at 502 nm. After gating of EGFP-positive cell population, DHE-positivity was measured with excitation at 488 nm and emission at 595 nm.

3.3 Determination of cell viability with MTT assay

Briefly, MTT-solution (Thiazolyl Blue Tetrazolium Bromide) was added to the cells in 96-well plates for last 2 hours. Next, the medium was removed and isopropanol-/HCl-solution was added. The dye formed was measured at 560 nm, and the absorbance was linear to the number of viable cells.

3.4 Western Blotting Analysis

In all Western Blotting experiments excluding the analysis of PARP, cells were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7,4), 1% NP-40, 0,25% natriumdeoxycholate, 150 mM NaCl, 1mM EDTA and protease inhibitors (Roche). For analysing PARP, cells were lysed in a buffer containing 62,5 mM Tris (pH 6,8), 6 M urea, 10 % glycerol, 2 % SDS, 5 % β-mercaptoethanol and 0,003 % bromophenol blue. In certain cases, phosphatase inhibitors were added as well. Protein concentrations were determined by BC Assay: protein quantitation kit. Proteins were separated using SDS-PAGE gels by electrophoresis and transferred to a nitrocellulose membrane, blocked either in Tris-buffered saline (TBS) and 5% skim milk or BSA, and incubated with primary antibodies diluted blocking buffer. Used antibodies were against BDNF, catalase, p-CREB, p-IκB-α, IL-6, NFκB (p65), PARP, SOD1, SOD2, spectrin, TrkB, p-TrkA/B, TRX2, XIAP or actin. Next day the membrane was incubated with a horseradish peroxidase conjugated secondary antibody, and detection was performed using the SuperSignal®West Pico Chemiluminescent Substrate. Quantifications were done using GelDoc (Bio-Rad).

3.5 Immunocytochemistry

PC6.3-cells in collagen-coated 24-well plates were fixed for 20 min RT using 4% paraformaldehyde (PFA), washed in phosphate-buffered saline (PBS) and permeabilized with 0,1% Triton-X-PBS for 5 min. After blocking with 5% BSA-PBS, the primary antibody for NFκB (p65) was added. The next day cells were incubated with Cy3™-conjugated secondary antibody and nuclei were stained using Hoechst 33258.

3.6 PCR and Light cycler

Total RNA was extracted and cDNA was synthesized from control and treated cells using dTT primers according to the manufacturers protocol (ThermoScript RT-PCR system). PCR was carried out by using the following primers: BDNF: FW 5'-TCG GTT GCA TGA AGG C-3' and RV 5'-GGT TTT CTT CGT TGG GC-3'; IL-6: FW 5'-CCA CCA GGA ACG AAA GT-3' and RV 5'-GCA TCA TCG CTG TTC ATA C -3'; SOD1: FW 5-CAA GCG GTG AAC CAG TTG TG-3 and RV 5'-TGA GGT CCT GCA GTG GTA C-3′; SOD2: FW 5'-GCC TGC ACT GAA GTT CAA TG -3' and RV 5'-ATC TGT AAG CGA CCT TGC TC -3'; TRX2: FW 5'-GGA CTT TCA TGC ACA GTG -3' and RV 5'-CGT CCC CGT TCT TGA T -3'. XIAP: FW 5'-TGC TGG ACT CTA CTA CA -3' and RV 5'-GAC TTG ACT CAT CCT GCG A -3'; β-actin from the same cDNA samples was used to control the total cDNA levels with the following primers: FW 5′-CAC ACT GTG CCC ATC TAT GA-3' and RV 5'-CCA TCT CTT GCT CGA AGT CT-3'. To detect human XIAP from transfected cells, we used human specific primers: FW 5′-CCC AAA TTC AAC AAA TCT-3' and RV 5-GAC TTG ACT CAT CTT GCA T-3'. Light Cycler was carried out for BDNF and actin by using the same primers as mentioned above.

3.7 Luciferase Assay

In luciferase assays cells were (co-)transfected with NFκB reporter or BDNF promoter I/IV constructs. *Renilla* luciferase (pRL-TK) was used to control transfection efficiency. In some experiments dominant negative (dn)-IκB plasmid was used to inhibit NFκB**.** Cells were lysed in lysis buffer containing 25mM Tris-phosphate (pH 7.8), 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol and 1% Triton X-100. For assaying the expression of *Renilla* and firefly luciferase we used the dual luciferase substrate and the activites were measured by luminometer (TD-20/20, Luminometer Turner Designs). The values for firefly luciferase were normalized to the *Renilla* luciferase activity.

3.8 Statistics

Paired t-test and one-way ANOVA were used for statistical comparisons of our experiments.

4. RESULTS AND DISCUSSION

4.1 XIAP decreases the production of intracellular ROS levels and cell death after oxidative stress (I + supplementary data)

The first aim was to study whether XIAP is able to have an effect on intracellular ROS levels, and cell death caused by ROS. It was noticed in our collaboration group using XIAP-TG mice that XIAP is able to protect against neuronal tissue loss after neonatal hypoxia-ischemia (Wang et al., 2004). There was also some evidence that XIAP might have an association with oxidative stress (Saito et al., 2004). We had a reason to believe that XIAP could reduce oxidative stress in our *in vitro* PC6.3-cell model as well, and we started to study this more closely. We used a construct with XIAP-EGFP fusion protein for transfections, so it was possible to count only the transfected, XIAP-overexpressing cells. DHE was used as an indicator to measure the amount of ROS, and the calculation of DHE-positive cells from GFP-positive cells was carry out by FACSAria. We observed that overexpression of XIAP reduces the generation of intracellular ROS in neuronal PC6.3-cells both alone and after exposure to oxidative stress by 100 μM xanthine (X) and 200 mU/ml xanthine oxidase (XO) for 1 hour (I ; see also chapter 4.2.2, figure 10). Parallel results concerning XIAP and oxidative stress was observed *in vivo* XIAP-transgenic (XIAP-TG) mice as well (Zhu et al., 2007).

The second aim was to confirm that XIAP is able to reduce cell death caused by ROS. Because the mechanisms of ROS leading to cell death are not fully understood, we also wanted to examine if the cell death is apoptotic or non-apoptotic in our model. With the MTT assay we perceived that overexpression of XIAP reduces cell death caused by ROS (X 100μM + XO 100 mU/ml; for 24h) (I). Then we investigated with few other methods whether cell death caused by ROS is apoptotic or non-apoptotic. Spectrin and PARP antibodies were used in Western Blotting analysis for determination of calpain and caspase activation after treatment with X+XO. Calpain is known to be activated in several necrotic and apoptotic conditions while caspase-3 is activated only in neuronal apoptosis (Wang, 2000). Both of these are cysteine proteases, and it is known that cleavage of spectrin occurs if caspase-3 and/or calpain are activated. PARP is a substrate of caspase-3 so it is cleaved if caspase-3 is activated. Our results indicated that X+XO stimulation does not activate caspase-3, but it activates calpain (figure 7a). This was the first support for the hypothesis that cell death caused by ROS (X+XO) is not apoptotic, at least in PC6.3-cells. BAF was used to inhibit caspases, and with the MTT assay we analysed if BAF was able to protect cells against xanthine and xanthine oxidase (figure 7b). Judging by it not having any protecting effects, X+XO must have other mechanisms besides caspases. This suggests support for the previous data that cell death caused by X+XO is not apoptotic. Last but not least, Hoechst-staining was used for determination of DNA content in single cell nucleus, revealing that some of the nuclei of cells treated with X+XO were condensed (figure 7c). Some of the nuclei had started to disintegrate with a structure that did not seem to be apoptotic, but because nuclear chromatin condensation occurred as well, cell death did not look like clear necrosis either. Maybe necroptosis then, or something in between necrosis and necroptosis? It is well-known that

XIAP can inhibit apoptotic cell death, but all of these data suggests that XIAP can protect cells against non-apoptotic cell death caused by oxidative stress as well.

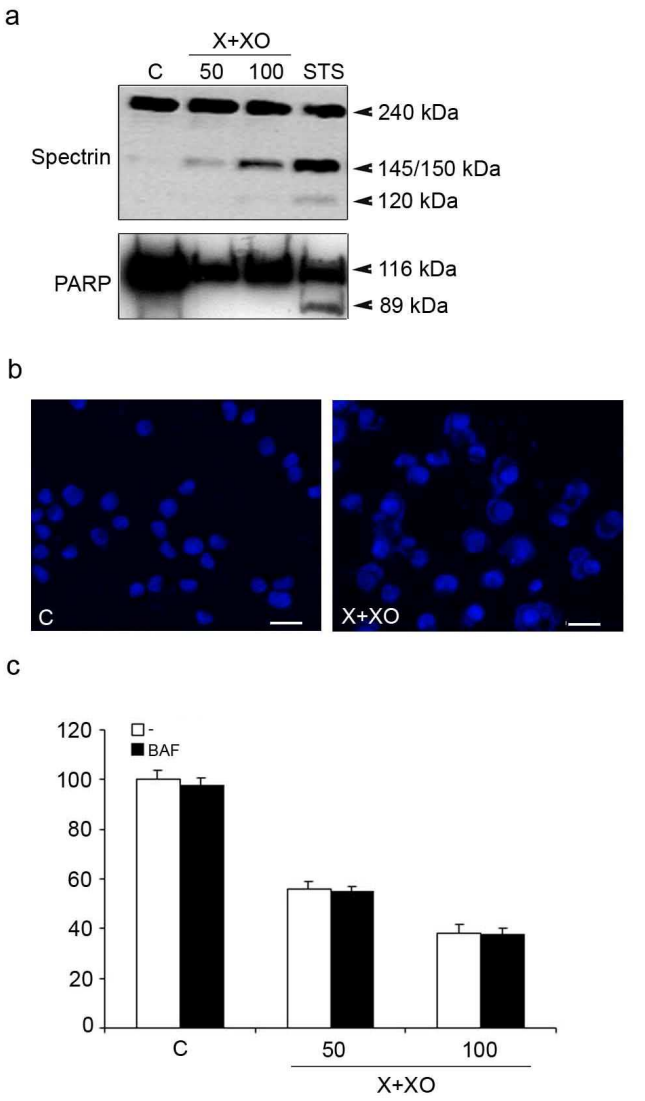

Figure 7. Cell death in our system is non-apoptotic (unpublished, supplementary data).
a) Cleavage of spectrin is visible if caspase-3 and/or calpain are activated, and cleavage of PARP occurs if caspase-3 is activated. 100 µM xanthine (X) and 50/100 mU/ml xanthine oxidase (XO) for 24h cleaved part of normal spectrin (240 kDa) to 145/150 kDa calpain product, but either 120 kDa caspase-3 product of normal spectrin or 89 kDa caspase-3 product of normal (116 kDa) PARP were not observable. 100 nM staurosporine (STS; 24h) was used as a positive control for activation of caspase-3 and calpain. b) Caspase inhibitor BAF was not able to inhibit cell death caused by 50/100 µM xanthine (X) and 50/100 mU/ml xanthine oxidase (XO). According to this, cell death caused by X+XO is not apoptotic. c) Hoechst staining showed that some of the cell nuclei treated with 100 µM xanthine (X) and 100 mU/ml xanthine oxidase (XO) o/n were condensed, but some have started to disintegrate. Scale bar = 10 µM.

4.2 XIAP increases mitochondrial antioxidant levels (I)

Based on previous findings, we were wondering whether XIAP might alter the regulation of antioxidant genes as well, and our next purpose was to find out whether this could be the case. This had not been shown before, but we noticed first that overexpression of XIAP increases both mRNA and protein levels of mitochondrial antioxidant SOD2 in neuronal PC6.3-cells (I ; see also chapter 4.2.2, figure 10). Parallel results concerning XIAP and intracellular antioxidants were published just a moment after our observations (Resch et al., 2008). Contrary to Resch and coworkers, we noticed that XIAP had no effect on the levels of SOD1 and catalase (I). Certainly, it has to be considered that they used XIAP -/- cells in their studies, and we used cells with overexpression of XIAP. In MEF-cells we also found out that overexpression of XIAP increased the protein levels of TRX2 (I). Our results indicated a preferential influence of XIAP particularly on the mitochondrial antioxidants: SOD2 and TRX2.

4.2.1 XIAP activates NFκB (I, II, Supplementary data)

Our next aim was to identify the mechanism behind the ability of XIAP to regulate SOD2 and TRX2. Previous investigations had shown that XIAP is able to affect various signalling pathways, including NFκB activity in endothelial cells (Hofer-Warbinek et al., 2000). In neuronal cells there was less evidence concerning the capability of XIAP to activate NFκB, but it was known that in neurons NFκB signalling is mainly protective (Middleton et al., 2000). We wondered whether XIAP would have an effect on the activation level of NFκB in neuronal cells as well, and studied this with three different methods in PC6.3-cells transfected with XIAP-constructs for 48 hours. The conclusion with all these methods was that overexpression of XIAP increases activation of NFκB (table 4, figure 8; see also chapter 4.2.2, figure 10). In addition to these, findings with PC6.3-cells, we also we noticed that overexpression of XIAP increases the activation of NFκB in E17 HC neurons as well (figure 8B).

Table 4. Overexpression of XIAP increases the activation of NFκB.

Method	How it was seen that XIAP activates NFκB?
Western Blotting	Protein IκBα was phosphorylated in XIAP transfected cells
Immunocytochemistry	Protein p65 was mainly in the nucleus in XIAP transfected cells
NFκB Luciferase Reporter Assay	Depending on the transfection efficiency, the activity of NFκB was about 10-20 times higher in XIAP-transfected cells compared to control cells

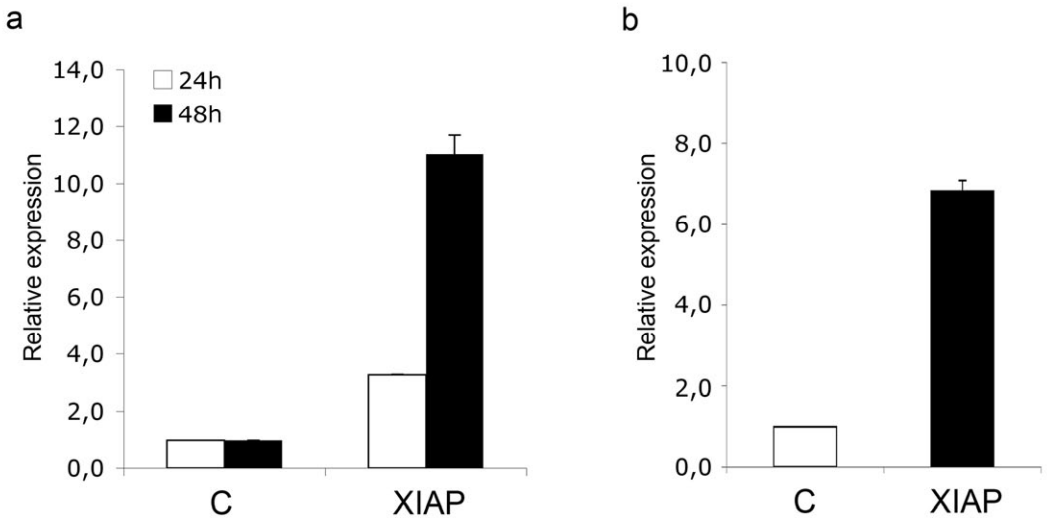

Figure 8. Overexpression of XIAP increases the activation of NFκB both in neuron-like PC6.3-cells and in E17 HC neurons. Cells were transfected with NFκB-Luciferase reporter plasmid together with control pcDNA3.1 (C) or XIAP-myc (XIAP) expression plasmid. Cotransfection with *Renilla* expression vector was used as a control to assay transfection efficiency. Results are fold induction of LUC activity after a) 24 and 48 h (PC6.3) and b) 7 days (E17 HC neurons) of transfection (unpublished, supplementary data).

Interaction between BIR1 domain of XIAP and TAB1 is known to be crucial for XIAP-induced activation of NFκB through TAK1 (Lu et al., 2007). The N-terminal domain of TAB1 interacts with the TAK1 kinase domain (figure 9). Activation of TAK1 on the other hand leads to phosphorylation of IKK and activation of NFκB (Wang et al., 1998). Researchers have shown that both activation of NFκB (or its *Drosophila* homologue Relish) and XIAP lead to inhibition of JNK signalling through proteasomal degradation of TAK1 (Park et al., 2004, Kaur et al., 2005). Prolonged activation of JNK is ROS-dependent, which supports the capability of XIAP, through NFκB, to reduce oxidative stress and perhaps modulate the expression of intracellular antioxidants**.** It would be interesting to investigate the roles of TAK1 and TAB1 in our model more closely in the future because it is known that XIAP activates NFκB through TAB1 and TAK1 (Lu et al., 2007).

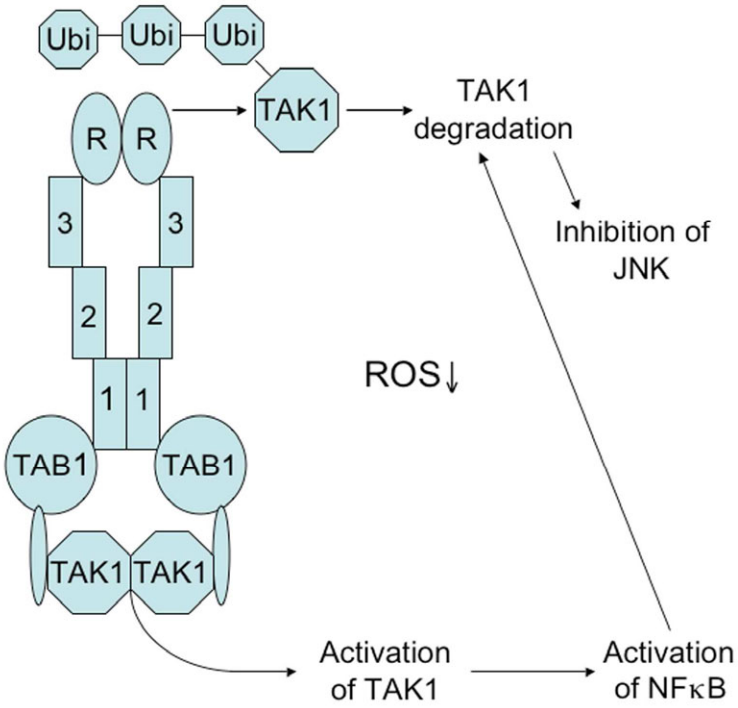

Figure 9. A schematic model for XIAP-mediated activation of NFκB. The BIR1 domain of XIAP is crucial for NFκB activation through TAB1 and TAK1. Activation of NFκB itself can induce degradation of TAK1 via upregulation of downstream target genes, such as XIAP. The RING domain of XIAP mediates the polyubiquitination of TAK1 and proteosomal degradation. Degradation of TAK1 leads to inhibition of JNK pathway.

4.2.2 The effect of XIAP on the levels of SOD2 and TRX2 is NFκB-dependent (I, III)

NFκB is known to have a role in SOD2 induction: p65 is capable of mediating the TNFα-induced SOD2 increase, and Heat-Shock Protein 25 (HSP25) regulates SOD2 through activation of NFκB (Maehara et al., 2000, Yi et al., 2002, Guo et al., 2003). We started to study if the effect of XIAP on SOD2 was mediated by NFκB, and first blocked the activity of NFκB with 2,5 mM ASA. Overexpression of XIAP did not increase the level of SOD2 if ASA was present (I), but because ASA can affect so many other cell signalling cascades as well, we wanted to confirm the observation with some other method as well. Secondly, we studied the same thing in immortalized embryonic fibroblast cell lines (1 and 2) from NFκB RelA/p65 knockout (KO) mice, which have been also used in earlier publications to study the NFκB pathway (Beg and Baltimore, 1996, Farhana et al., 2005). We observed in both of the p65-/- cell lines that the rise of SOD2 is p65-dependent: overexpression of XIAP was not able to increase the protein levels of SOD2 in cells lacking p65 (-/-), but the increase was visible in control fibloblast

cell line 3T3 (I). This data suggests that NFκB is involved in the ability of XIAP to increase SOD2 (figure 10).

We also investigated whether the increase of mitochondrial antioxidant protein TRX2 by XIAP is NFκB dependent. Earlier, it has been shown that TRX2 inhibits TNFα-induced ROS generation, downstream NFκB activation and apoptosis (Hansen et al., 2006). We noticed that overexpression of XIAP increased the amount of TRX2 in control 3T3-cells but not in p65-/- cell lines (I), so NFκB is involved in this effect of XIAP (figure 10).

One more thing we noticed was that the basic levels of SOD2 and TRX2 are lower in cells lacking p65 than in control cells (III). This observation concerned only these mitochondrial antioxidants, and e.g. SOD1 was not increased by XIAP (I), and the amount of it was not lower in p65-/- cell lines (data not shown). Actually the protein levels of SOD1 seemed to be approximately higher in p65-/- cell lines than in control 3T3 cell line. This brings to mind whether this could be some kind of a compensatory mechanism of the p65-/- cells?

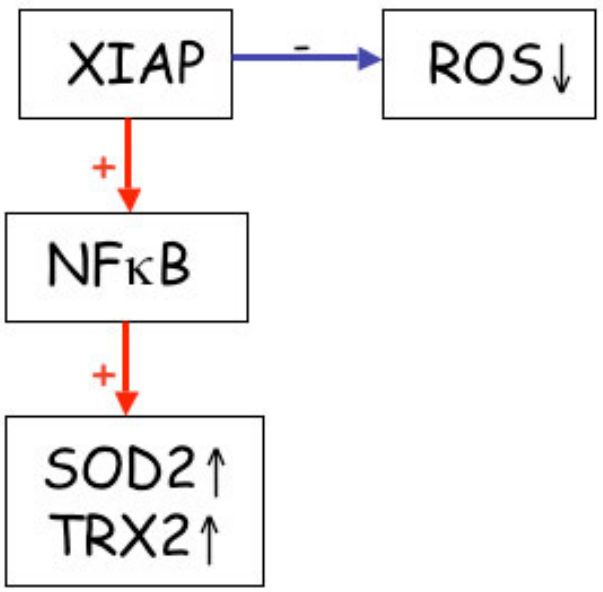

Figure 10. Summarizing figure: XIAP reduces oxidative stress and increases the levels of mitochondrial antioxidants (SOD2 and TRX2) through increased activation of NFκB.

4.3 XIAP increases the amount of BDNF and induces BDNF gene activation through NFκB (II)

Using Affymetrix microarrays to analyse gene expression, our collaborator group had observed that the mRNA of neurotrophic factor BDNF was upregulated in brain tissue of transgenic (TG) XIAP mice with overexpression of XIAP in neurons. With Western Blotting analysis we noticed that the protein levels of BDNF were also higher in the brains of XIAP-TG mice than in control brains (II). The connection of XIAP and BDNF had not been observed before, but the inhibition of XIAP activity has been shown to prevent GDNF-mediated neuroprotective effects (Perrelet et al., 2002).

Our next purpose was to study the mechanisms behind the effect of XIAP on BDNF by using E17 HC neurons. First, we confirmed that we are able to see the same effect of XIAP to BDNF *in vitro* as well, and noticed that overexpression of XIAP (2-7 days) increased both mRNA and protein levels of BDNF in E17 HC neurons (II). We also made a notification that in the presence of XIAP-antagonist embelin, XIAP-myc transfection did not affect to BDNF. Experiments made with neurons treated with XIAP silencing RNA (siRNA) show that both mature BDNF and its precursor pro-BDNF were downregulated when XIAP was silenced (II). This indicates that XIAP is able to affect BNDF already in its synthesis phase.

Then, we investigated the gene activity of BDNF by using BDNF promoters linked to luciferase reporter in neuronal PC6.3-cells. Our observation was that both BDNF promoter I and IV activities were increased if XIAP was overexpressed (II). In contrast to this, downregulation of XIAP with siRNA decreased BDNF I and IV promoter activities (II). dn-IκB construct almost blocked the XIAP increased activation of BDNF promoter IV, and reduced the increase of promoter I activity (II). This indicates that NFκB activation induced by XIAP affects transcription of promoters I and IV BDNF. Previous studies have shown the presence of a binding site for NFκB in the BDNF promoter IV, that mediates the stimulation of BDNF induced by N-methyl-d-aspartate receptor (NMDAR) (Jiang et al., 2008). NFκB-binding element has also been identified in the BDNF promoter I which is involved in the regulation of BDNF expression by kainic acid (KA) (Lubin et al., 2007). These binding sites may be targets for the NFκB-dependent regulation of the BDNF gene activity by XIAP as well.

4.3.1 Other proteins involved in the regulation of BDNF by XIAP

We also desired to examine if BDNF was regulated through other proteins induced by XIAP. It was known that XIAP is able to regulate interleukin-6 (IL-6), at least in endothelial cells, via stimulation of NFκB and MAP kinase signalling pathways (Resch et al., 2006). IL-6 has been connected to neuroprotection and neuronal survival after ischemic and other injuries (Ohtaki et al., 2006, Fritzenwanger et al., 2007). However, not much was known about the protective genes affected by cytokines in the brain. We clarified that XIAP does this in neuronal PC6.3-cells and in E17 HC neurons as well (II). We also studied if IL-6 was associated with the ability of XIAP to regulate BDNF and

NFκB signalling. Our observation was that IL-6 increased the protein levels of BDNF in E17 HC neurons, and IL-6 antibodies blocked the upregulation of BDNF caused by XIAP (II). Anyhow, IL-6 did not alter the activation of BDNF Promoter activities, as XIAP itself did (II). Altogether, IL-6 is related to interconnection of XIAP and BDNF, but the precise mechanism behind this remains to be studied in the future. It also would be worth to clarify whether XIAP would increase IL-6 if NFκB was blocked - at least XIAP was not able to activate BDNF promoters I and IV completely if dn-IκB was present.

Using Affymetrix microarrays, our collaborator group also noticed that the expression of BDNF receptor TrkB was increased in the XIAP overexpressing neurons in TG-XIAP mice compared to controls. We observed the same elevation of TrkB by Western Blotting analysis in XIAP-transfected E17 HC neurons compared to control cells (II). The exact mechanism by which XIAP increases TrkB here and whether it involves NFκB, remains to be studied. We also studied the levels of activated (phosphorylated) TrkB receptors in E17 HC neurons using p-TrkA/B antibodies. Only a small amount of TrkA is found in HC neurons, so the results can be considered as indicators of TrkB activation. Overexpression of XIAP increased the levels of p-TrkB in E17 HC neurons (II), which suggests that the whole BDNF-TrkB-system is more activated in XIAP-overexpressing neurons. Following the phosphorylation of TrkB, BDNF can stimulate various signalling cascades which regulates transcription factors such as the CREB protein (Huang and Reichardt, 2003). Activation (phosphorylation) of CREB on the other hand is known to regulate the transcription of other genes including BDNF, and CREB is usually related to same pathological conditions as BDNF. We also noticed that the levels of phosphorylated CREB protein (p-CREB) were increased in HC neurons after the XIAP overexpression (II). Presence of BDNF scavenging TrkB-soluble receptor bodies (TrkB/Fc chimeras) prevented the increase of p-TrkB and p-CREB induced by XIAP-transfection, which suggests that our findings are originating from the increased level of BDNF caused by overexpression of XIAP.

Altogether, these studies show that XIAP has an influence on the expression of the neurotrophic molecules BDNF and IL-6. XIAP affects neuronal signalling via the upregulation of p-TrkB and p-CREB. Our results expose a novel cytokine network in the brain, which involves XIAP, BDNF, and IL6 interconnected through the NFκB system (figure 11), which can influence several aspects of neuronal survival. Because BDNF has a role in diseases where oxidative stress is also involved, such as Huntington's Disease, it is really fascinating that XIAP can have a positive effect on both BDNF signalling and oxidative stress.

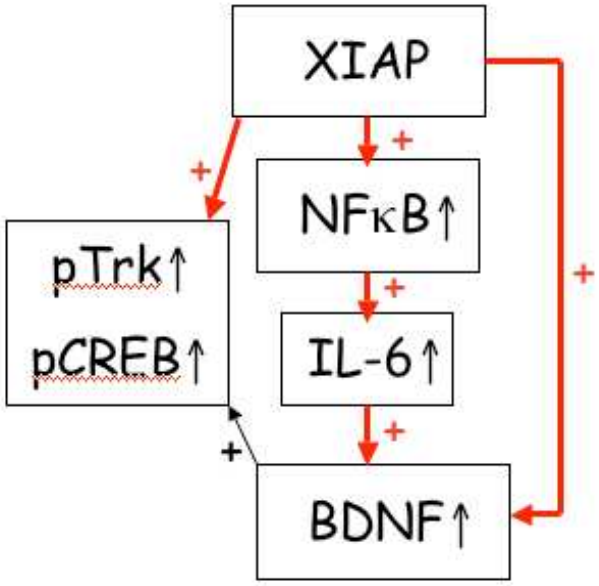

Figure 11. Summarizing figure: XIAP affects BDNF levels and gene activation through NFκB. The levels of IL-6 are also increased by XIAP, and NFκB is known to have a role in the interconnection of XIAP and IL-6 (Resch et al., 2006). IL-6 seems to be somehow involved in interconnection of XIAP and BDNF as well, while it increases the amount of BDNF. In addition, XIAP increases the activation of BDNF-TrkB-system and CREB.

4.4 Oxidative stress is involved in HD (III)

Changes in mitochondria and other organelles with increased oxidative stress have been linked to HD (Gil and Rego, 2008, Lipinski and Yuan, 2004). Our own group has previously shown that N-terminal huntingtin proteins with extended polyQ repeats induce ER stress (Reijonen et al., 2008). On the other hand, ER is able to produce ROS during normal cell functions, such as metabolism and protein folding (Finkel and Holbrook, 2000, Jones, 2008). In addition to ROS, some transcription factors or their co-activators which have been connected to stress reactions, such as CREB, NFκB, PGC1α, and p53, have been noticed to be affected by CAG expansion (Steffan et al., 2000, Roze et al., 2008, Cui et al., 2006, Weydt et al., 2006). In our own studies, we had previously connected NFκB to oxidative stress and intracellular antioxidants, which raised the question, that maybe oxidative stress together with NFκB may have a role in HD?

The target of this part of the study was to investigate oxidative stress, intracellular antioxidants and NFκB in our *in vitro* model of HD. We observed in PC6.3-cells transfected with expression plasmids encoding different polyQ repeats of huntingtin exon-1, that the amount of ROS increases while the number of CAG-repeats expands (III; figure 12). Cells transfected with the construct containing 18 polyQ ("normal amount") repeats show about the same ROS levels as control cells, while ROS levels of cells transfected with the mutant, 120 polyQ repeats containing construct were about threefold higher. DHE was used as an indicator to measure the amount of ROS, and the calculation

of DHE-positive cells from GFP-positive cells was carried out by FACSAria. The protein levels of intracellular antioxidants SOD2, SOD1, TRX2, and catalase decreased in cells expressing mutant N-terminal fragment huntingtin proteins compared to control cells as measured with Western Blotting analysis (III; figure 12). The same decrease was visible for the protein levels of NFκB as well. NFκB activation was quantified with the NFκB reporter (luciferase) assay, and the activation of NFκB was about half in cells transfected with disease-including 75 Q-containing huntingtin compared to cells transfected with "normal" amount (17 Q) containing huntingtin (III). In the previous studies we noticed that at least mitochondrial antioxidants were increased by XIAP through NFκB, and the amount of ROS were decreased by XIAP. It would be worth finding out whether the induction of NFκB activity e.g. by XIAP inhibits the accumulation of ROS and/or reduction of antioxidant levels caused by mutant huntingtin? Reduced XIAP levels have been linked to HD both in brain-derived cells expressing mutant huntingtin, and in post-mortem HD brain tissue (Goffredo et al., 2005). And although XIAP would not be affected directly in HD or vice versa, it is certainly possible that modulation of XIAP can vicariously have positive effects in HD by reducing oxidative stress, and in this way protecting cells.

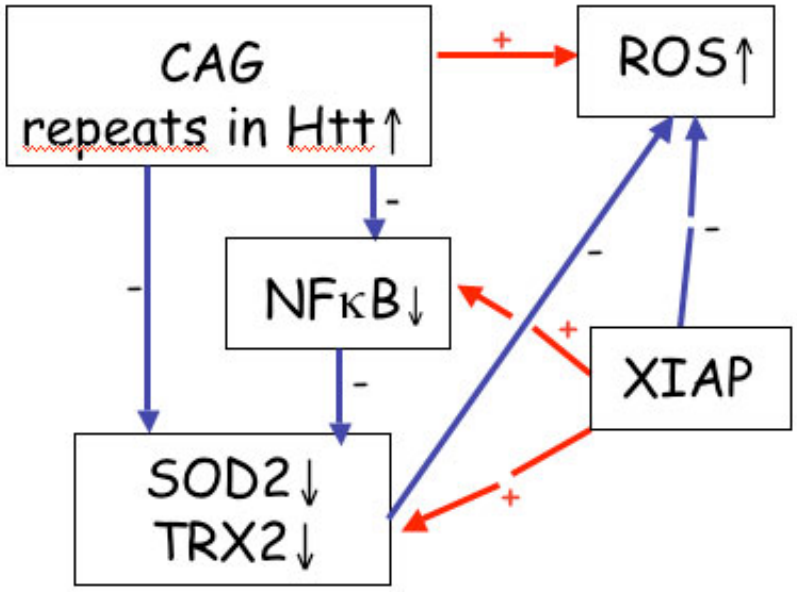

Figure 12. Summarizing figure: the amount of ROS increases, and intracellular antioxidants (i.e. SOD2 and TRX2) and NFκB decreases, while the number of CAG-repeats multiplies. Broken lines present the data according to the previous studies: XIAP reduces oxidative stress and increases the amount of SOD2 and TRX2, and activation of NFκB.

4.5 RSV pretreatment reduces the production of intracellular ROS levels and cell death after oxidative stress (IV)

Previously, RSV has been shown to increase antioxidant levels in cultured PC12 cells and SOD2 in human fibroblasts (Chen et al., 2005, Robb et al., 2008). RSV has also shown to have neuroprotective effects against metabolic dysfunction induced by mutant huntingtin (Parker et al., 2005). Based on the antioxidant properties of RSV, we started to investigate the mechanisms more closely. First we confirmed that RSV pretreatment is able to reduce oxidative stress also in our system. We pretreated neuronal PC6.3-cells with 50 or 75 μM RSV, and 30 min after that stimulated cells with 100 μM xanthine (X) and 200 mU/ml xanthine oxidase (XO) for 1 hour. We noticed that RSV reduced generation of intracellular ROS both alone and after exposure to oxidative stress (figure 13). DHE was used as an indicator to measure the amount of ROS, and the calculation of DHE-positive cells was carried out by FACSAria. We also examined cell viability with the MTT assay following 24 h X + XO (100 μM + 200 mU/ml) treatment, and found that both RSV concentrations (50 μM and 75 μM) reduced cell death caused by X + XO about 25 %.

4.5.1 RSV increases mitochondrial antioxidants and XIAP, and NFκB may contribute to this (IV)

Because RSV decreased the levels of ROS in neuron-like PC6.3-cells, we had a reason to believe that RSV may have an influence on intracellular antioxidants as well. Earlier, it has been shown that RSV can induce SOD2 in human fibroblasts (Robb et al., 2008). We noticed that RSV-treatment (50-75 μM; 24 h) increases mRNA and protein levels of mitochondrial antioxidants SOD2 and TRX2, in neuronal PC6.3-cells (IV; figure 13). The increase was even higher if xanthine and xanthine oxidase were added. We also investigated if RSV is able to influence XIAP levels, and observed, that RSV increased both mRNA and protein levels of it (IV; figure 13). After a longer treatment (72 h), the effect of RSV on SOD2 and TRX2 was even stronger, and visible already with smaller concentrations (10 μM and 25 μM).

Many of the antioxidants are known to be regulated by the NFκB system. We have noticed in our previous studies that the responses of SOD2 and TRX2 to XIAP are mediated by NFκB activation (I). Because of this, we were considering if the effects of RSV on mitochondrial antioxidants are mediated by NFκB as well? It has been reported that RSV suppresses NFκB e.g. in carcinoma cells (Manna et al., 2000, Banerjee et al., 2002). Anyway, the connection of RSV and NFκB has not been studied that much in neuronal cells. It is still known that RSV can act differently in different cell types (Shakibaei et al., 2009). The same RSV concentration can be protective to some cells and harmful to others, and high concentrations may induce apoptosis whereas low concentrations may promote cell survival (Jang et al., 1997, In et al., 2006, Shakibaei et al., 2009). The precise timing for RSV treatment is also worth to consider.

The NFκB Luciferase Reporter Assay showed that after 24 h, 50 µM RSV had a small positive effect on the activity of NFκB, but 75 µM RSV did not (IV). After 48 h, both 50 µM and 75 µM RSV increased the activity of NFκB about 2.5 fold. It is possible that the activation of NFκB is involved in the increase of antioxidants mediated by RSV (figure 12). Apart from NFκB, other signalling pathways may be involved in counteracting oxidative stress. It has been shown that RSV can activate AMPK, SIRT-1, and PGC-1α (Baur et al., 2006, Lagouge et al., 2006). It remains to be studied whether some of these pathways are affected by RSV in PC6.3-cells. PGC-1α has previously been linked to intracellular antioxidant proteins, and has shown to be regulated by RSV (St-Pierre et al., 2006, Lagouge et al., 2006).

Altogether, our observations present that RSV protects neuron-like PC6.3-cells from cell death caused by oxidative stress, and RSV reduces the generation of intracellular ROS. The NFκB signalling pathway may be involved in this. RSV is worth to consider as a drug candidate because it is known to cross the blood-brain barrier (BBB), and increased ROS levels are presented to be involved in neurodegenerative diseases such as Huntington's, Parkinson's, and Alzheimer's diseases(Barnham et al., 2004, Henrotin et al., 2003, Rego and Oliveira, 2003, Baur and Sinclair, 2006). Certainly the clinical use of RSV will need careful analysis of its kinetics, actions and pharmacological and toxicological profiles.

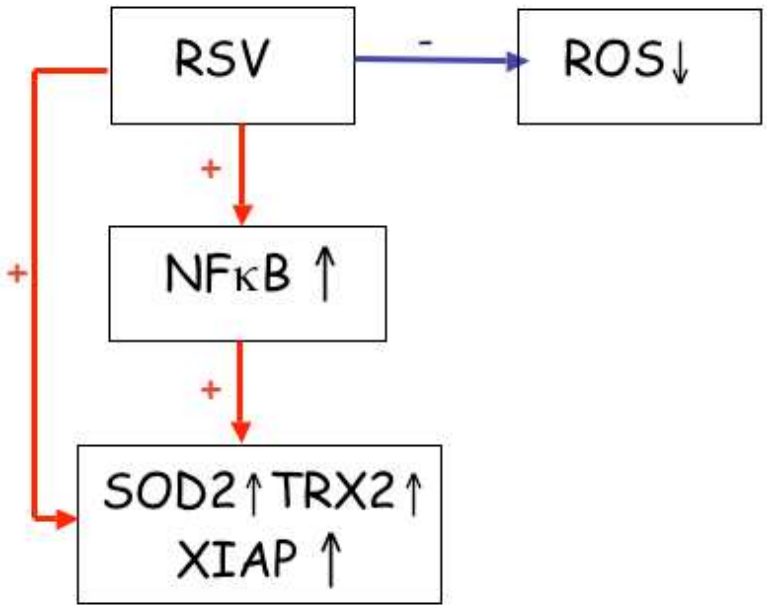

Figure 13. Summarizing figure: RSV decreases the amount of ROS, and increases the mitochondrial antioxidants (SOD2 and TRX2) and XIAP. RSV is also able to activate NFκB if the dose and timing are optimal. SOD2, TRX2 and XIAP are downstream target genes of NFκB, so it is possible that the activation of NFκB is involved in the increase of antioxidants mediated by RSV as well.

5. CONCLUSIONS AND FUTURE POSSIBILITIES

The first aim of this work was to clarify the possible role and mechanisms of anti-apoptotic protein XIAP in cell death caused by oxidative stress in neuronal cells. We observed that XIAP reduces oxidative stress and cell death caused by ROS in neuronal cells. Because oxidative stress is the result of an imbalance between pro- and antioxidant homeostasis, we had reason to believe that XIAP might alter the regulation of antioxidant genes in neuronal cells as well. This had not been shown before, and our next purpose was to clarify whether XIAP has this effect?

Secondly, we showed that XIAP activates NFκB in neuronal cells, and increases both mitochondrial antioxidants (SOD2 and TRX2) and BDNF signalling through NFκB. Because it is known that XIAP activates NFκB through TAB1 and TAK1, it would be interesting to investigate the roles of TAK1 and TAB1 in our model more closely in the future. Modulation of XIAP is also an interesting possibility to consider in various therapies to reduce cell injuries caused by increased oxidative stress.

Thirdly**,** we saw in the *in vitro* HD model that the amount of ROS increases while the number of CAG-repeats expands. The protein levels of intracellular antioxidants (SOD1, SOD2, TRX2, and catalase) and NFκB also decreased in cells expressing mutant huntingtin compared to control cells. These results support that in HD there is an imbalance between pro- and antioxidant homeostasis within the cells. Because NFκB also seemed to be involved, what naturally came to mind was: would it be possible to affect HD in a positive way by modulating XIAP? XIAP not only decreases oxidative stress, but it also regulates BDNF signalling through NFκB, and deprivations in BDNF gene transcription has been demonstrated to be in connection with the mutation in the HD gene as well. The role of XIAP in our HD cell model remains to be studied closely in the future.

Lastly, we studied whether RSV is able to protect neuronal cells against oxidative stress, and noticed that it reduces oxidative stress and cell death caused by ROS. RSV also increased the amount of mitochondrial antioxidants (SOD2 and TRX2) and XIAP. This effect was even stronger if the treatment was longer. We also observed that RSV is able to activate NFκB if the dose and timing are optimal. Because RSV is known to cross the blood-brain barrier, it would be an interesting compound to examine as a drug candidate in disorders where oxidative stress is involved. Certainly, the clinical use of it will need a lot of studies to analyse the kinetics, actions and pharmacological and toxicological profile of it.

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Helsinki, September 2010

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